Allelic Transcripts Dosage Effect in Morphologically Normal Ovarian Cells from Heterozygous Carriers of a BRCA1/2 French Canadian Founder Mutation

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Abstract
We hypothesized that the transcriptome of primary cultures of morphologically normal ovarian surface epithelial cells could be altered by the presence of a heterozygous BRCA1 or BRCA2 mutation. We aimed to discover early events associated with ovarian carcinogenesis, which could represent putative targets for preventive strategies of this silent killer tumor. We identified the first molecular signature associated with French Canadian BRCA1 or BRCA2 founder mutations in morphologically normal ovarian epithelial cells. We discovered that wild-type and mutated BRCA2 allelic transcripts were expressed not only in morphologically normal but also in tumor cells from BRCA2-8765delAG carriers. Further analysis of morphologically normal ovarian and tumor cells from BRCA1-4446C>T carriers lead to the same observation. Our data support the idea that one single hit in BRCA1 or BRCA2 is sufficient to alter the transcriptome of phenotypically normal ovarian epithelial cells. The highest level of BRCA2-mutated allele transcript expression was measured in cells originating from the most aggressive ovarian tumor. The penetrance of the mutation and the aggressiveness of the related tumor could depend on a dosage effect of the mutated allele transcript. Cancer Prev Res; 1–13. ©2012 AACR.

Introduction
Approximately 15% to 20% of breast and ovarian cancers occur in a familial context and approximately 5% to 7% exhibit a clear hereditary pattern. The identification of 2 major susceptibility genes, BRCA1 and BRCA2, has led to remarkable advances in our understanding of familial breast and ovarian cancers (1, 2). Greater susceptibility to breast cancers (particularly in women aged 50 years or younger), ovarian adenocarcinomas, and fallopian tube cancers are observed among female carriers of BRCA1 and BRCA2 mutations (3–6), whereas male carriers are at higher risk of developing breast or prostatic cancers (7). Thus, because of a strong variability in the penetrance and expressivity of BRCA1 and BRCA2 mutations (8), cancer risk prediction in unaffected mutation carriers remains a significant challenge. Inter- and intrafamilial phenotypic heterogeneity are recorded for the same mutation (9). Ethnicity may also contribute to the phenotypic variation noted in BRCA1 and BRCA2 families (10), making it increasingly difficult to offer adequate genetic counseling to mutation carriers.

In fact, the only efficient prevention alternative that high-risk women have at their disposal is prophylactic surgery. The quasi-absence of markers for the early detection of ovarian cancers remains a major limitation in cancer prevention programs (11). Improving our knowledge of the early events involved in inherited ovarian carcinogenesis is invaluable for the development of new diagnostic strategies and the identification of innovative preventive and therapeutic targets.

Various published data indicate the existence of a distinct phenotype associated with heterozygosity in morphologically normal ovarian surface epithelium (NOSE) cells, as reviewed in the work of Wong and Auersperg (12). After several passages in culture, cells from donors with a strong...
family history of cancer (FH-NOSEs) acquire characteristics that resemble those of epithelial ovarian tumor cells. They preserve their epithelial morphology but lose their capacity to respond to epithelial–mesenchymal transition signals in culture in contrast to NOSE cells from women with no familial history of cancers (NFH-NOSEs) which are highly responsive to these signals (12). Constitutive expression of E-cadherin, elevated CA125 expression, telomeric instability, coexpression of the hepatocyte growth factor (HGF), and its Met receptor, as well as signaling activation occur in cultured FH-NOSEs. SV-40–transformed NFH-NOSEs acquire greater self-sufficiency and similar characteristics (12). A specific phenotype has been reported in the BRCA2 heterozygous chicken B cell line DT40, characterized by reduced cell proliferation rates, increased cell death, greater sensitivity to DNA-damaging agents, and decreased RAD51 foci formation after irradiation. This phenotype is strikingly similar to the one described in the BRCA2 human ovarian tumor cell line TOV-81D, which retains both the normal and mutant copy of BRCA2 (13, 14). Thus, it appears that, even in the context of cancer, the presence of a BRCA2 heterozygous mutation might be associated with distinct phenotypic changes.

With all these observations in mind, we hypothesized that cells originating from morphologically normal ovarian cell display a distinct expression profile in carriers. The aim of this study was first to identify the molecular signatures associated with either BRCA1 or BRCA2 mutations in NOSEs. Moreover, the molecular signature associated with transformation from morphologically normal to tumor cells was investigated among BRCA1 and BRCA2 mutation carriers. After discovering altered transcriptomes in morphologically normal cells, we validated our microarray results on 4 candidate genes by quantitative real-time PCR (q-RT-PCR). We also developed allele-specific transcript quantification on BRCA1 and BRCA2 in mutation carriers to measure the mutated allele transcript, to identify a putative dosage effect.

As we were completing our q-RT-PCR validation, the Knudson team (15) published their findings on breast and ovarian morphologically normal cells, showing an altered gene expression associated with BRCA1/2 mutations distinct from the French Canadian founder mutations described in our study. Despite differences in terms of specific genes that are modulated, both their study and ours point to a clear effect of BRCA mutations on the transcriptome of normal cells, supporting the great interest of these findings for the scientific community.

Samples, Patients, and Methods
Cell cultures and clinical materials
After obtaining appropriate consent, normal samples were collected after surgeries conducted at the Centre Hospitalier de l’Université de Montréal (CHUM)–Hôpital Notre-Dame (Montréal, QC, Canada). Primary cultures of NOSEs were established, as described previously (16, 17), at the Fonds de la recherche en santé du Québec (FRSQ) Bank of the CHUM. Briefly, NOSEs were obtained by gently scraping, 2 to 3 times with a rubber scraper, the ovarian surface epithelium of the surgical specimen provided by the pathologist. The cells collected were then seeded in standard OSE medium consisting of 50:50 medium 199:105 (Sigma) supplemented with 15% FBS, 2.5 μg/mL ammonotitcin B, and 50 μg/mL gentamicin. Additional NOSE primary cultures were established with a procedure modified by Kruk (16). After rinsing, an explant of the surgical specimen was placed in 35-mm culture dishes and held surface down so that the ovarian surface epithelium was maintained in a small amount of medium in contact with the bottom of the culture dishes, allowing the NOSEs to colonize the plastic. The cells were then passaged at 80% of confluence (17). A subgroup of NOSEs was cultivated in a modified medium, that is, the standard OSE medium supplemented with EGF, hydrocortisone, insulin, and bovine pituitary extract (18). Finally, tumors of ovaries (TOV) were established in OSE medium supplemented with 10% FBS (17). Primary cultures of 9 NOSEs and 4 TOVs were included in our microarray analysis, whereas a more extensive set was used to validate the microarray pattern of expression. Among the 18 NOSEs, 8 were from women who underwent surgery to treat a benign gynecologic pathology located outside the annexial tract and 10 were from women who underwent prophylactic bilateral salpingo-oophorectomy (3 BRCA1 and 7 BRCA2 mutation carriers). Finally, TOVs were obtained from 3 BRCA1 and 2 BRCA2 mutation carriers. All donors were of French Canadian descent. An extensive description of the clinical data, BRCA1/2 mutation status, culture characteristics, and analyses associated with each sample is provided in Tables 1 and 2. The unique cell line used in this study, TOV-81D, has previously been established and described by our laboratory (13, 14). All extractions were conducted within fewer than 6 months from resuscitation of frozen stocks established at the time of their original description.

RNA extraction
Total RNA was extracted with TRIzol™ reagent (Gibco/BRL, Life Technologies Inc.). RNA was extracted directly from homogenized cells grown to 80% confluence in 100-mm Petri dishes. RNA quality was monitored with a 2100 Bioanalyzer and RNA 6000 Nano LabChip kit (Agilent Technologies) according to the manufacturer’s protocol.

Reverse transcription
Reverse transcription was conducted on 1 μg of total RNA with the SuperScript First-Strand Synthesis System (Invitrogen Canada Inc.) and random hexamers, according to the procedure recommended by the manufacturer. cDNA samples were diluted at 1:3 or 1:6 in water before q-RT-PCR. Three microliters of this dilution were used for each experiment.

Microarray analysis
Expression profiles of selected samples were identified with Affymetrix HuFl GeneChips. RNA treatment,
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<td>NOV-2075G KMP/MM</td>
<td>L NOSE p6 8XT</td>
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Abbreviations: FCH, familial cancer history; KMP, Kruk modified procedure; L, left ovary; MA, microarray; NA, not applicable; R, right ovary; U, Unknown laterality.
hybridization assays, and microarray scanning were conducted at the McGill University and Genome Quebec Innovation Centre (Montreal, QC, Canada), as described previously (19). The expression data were analyzed on the Bioconductor platform, an open source, and development software project for the assessment and comprehension of genomic data, primarily based on the R programming language (20). Raw signals generated by quantifying each sample with HuFL were reprocessed [background subtraction, normalization, and log2 transformation by the RMA (robust multiarray averaging) package (ref. 21)]. Raw and processed gene expression microarray data of this study are available for public access through the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database (22) using the GEO series accession number GSE34928.

Supervised analysis

Five \textit{a priori} classes of samples were defined and 5 different contrasts were computed: NF-NOSEs (NOSEs from noncarriers) versus F-NOSE1 (NOSEs from carriers of a \textit{BRCA1} mutation), NF-NOSEs versus F-NOSE2 (NOSEs from carriers of a \textit{BRCA2} mutation), F-NOSE1 versus F-NOSE2, F-NOSE1 versus F-TOV1 (TOVs from carriers of a \textit{BRCA1} mutation) and, finally, F-NOSE1-2 (union of the F-NOSE1 and F-NOSE2 classes) versus F-TOV1-2 (union of the F-TOV1 and F-TOV2 classes; Fig. 1).

Differentially expressed probe sets (DEPS) were identified on the basis of a moderated t-statistic computed with the Limma package for each contrast of 2 \textit{a priori} classes of samples. Standard errors were moderated across all probe sets measured with the HuFL microarray, that is, shrunk toward a common value with a simple Bayesian model (23). The Benjamini and Hochberg (BH) correction method was applied to control the false discovery rate across all genes. Probe sets with $q$-values (adjusted BH $P$ values) $\leq 0.05$ for most of the comparisons (Fig. 1, volcano plots) and $\leq 0.2$ for F-NOSE1-2 versus F-TOV1-2 contrast (data not shown) were retained as candidates. Significant statistical results for a set of 4 candidate genes are summarized in Supplementary Table S1.

Hierarchical clustering of the samples, based on DEPS identified for each contrast, was conducted thereafter with the “hclust” function of the “stats” R package to confirm whether the identified candidate probe sets were able to properly classify corresponding samples. Euclidean distances and the complete linkage method served to define clusters (Fig. 1, heat maps).

\textbf{BRCA1, BRCA2, and 4 candidate gene transcripts were quantified} by q-RT-PCR, with the Rotor-gene 3000 Real-Time Centrifugal DNA Amplification System (Corbett Research). The thermal cycling conditions were as follows: after 1 cycle of decontamination (50°C, 2'), and denaturation (95°C, 10'). cDNA was amplified for 45 cycles (95°C, 30', 60°C, 40'). Primers and TaqMan probes were designed with the Primer3 online tool (24), so that the selected amplicon corresponded to a transcript variant region of the candidate genes recognized by the HuFL array DEPS.
probes were synthesized by IDT (Integrated DNA Technologies) with TaqMan biochemistry. Target gene probes were coupled to the 6-FAM reporter, whereas the normalizer gene probe was coupled to the HEX reporter. Black Hole Quencher-1 was used as a quencher. All reactions were undertaken with Platinum Quantitative PCR SuperMix-UDG (Invitrogen Canada Inc.).

TBP (TATA-binding protein) served as a normalizer gene in each run. All reactions were in duplex with the normalizer gene. TBP was chosen as a reference gene, as its transcript showed stable expression within sporadic ovarian tumors (25) and did not show any significant difference in the present microarray analyses. Our further q-RT-PCR results confirmed its appropriateness as a reference gene. Reactions were optimized for better efficiency without interference between the gene of interest and normalizer gene amplification and for reproducibility of the results. A solution of 0.1 to 0.2 μmol/L of primers (forward and reverse) and probes and 2 mmol/L of MgCl2 was added to the 1× SuperMix (Invitrogen Canada Inc.) to obtain a final volume of 15 μL. A standard curve with serial dilutions of a cDNA, amplified by PCR and then purified with the QIAquick PCR Purification Kit (Qiagen Inc.), from cell lines TOV-112D, OV90, or TOV-21G was established for each gene of interest. A calibrator sample from the standard curve served as a control in each experiment. The gene of interest (target gene) to the normalizer gene (reference gene) relative expression ratio was measured by the Pfaffl algorithm:

\[ R = \left( \frac{E_{\text{target}}}{E_{\text{ref}}} \right)^{\frac{\Delta C_p(\text{target})}{\Delta C_p(\text{ref})}} \]

where \( R \) represents the relative expression, ratio \( E \) is the PCR efficiency, and \( \Delta C_p \) (mean control – mean sample) is the difference between the \( C_p \) (crossing point at a given threshold) of an unknown sample and a control (26). Two independent cDNAs were quantified. Each experiment was run in duplicate or triplicate for each independent cDNAs. The primers and probes used in these experiments are listed in Supplementary Table S4.

Allele-specific quantification

The BRCA1-4446C>T and BRCA2-8765delAG allelic transcript (wild-type and mutated) were quantified by

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**Figure 1.** A–D, volcano plots and heat maps for each contrast computed by Limma. Volcano plots (left chart) represent the log_{2} fold change between the 2 classes of samples versus log odds (or B-statistic), that is, the odds that each probe set, represented by a dot, is differentially expressed. Significant DEPS (\( q \leq 0.05 \)) are represented by a red dot on the plot. Heat maps with hierarchical clustering of the samples (right charts). Probe sets with a \( q \)-value \( \leq 0.05 \) were used to draw the heat maps and hierarchical trees. Each column represents a sample; each line, a probe set; and each tile, the expression level of the probe set. Color code: blue, overexpression; orange, underexpression. Color intensity is correlated with the corresponding probe set level of expression. Significant DEPS allowed clear segregation between the 2 classes for each contrast under study.
q-RT-PCR. The thermal cycling conditions were after a cycle of decontamination (50°C, 2'), and denaturation (95°C, 10'), cDNA was amplified for 45 cycles (95°C, 30', 50°C, 40' for BRCA1-4446C>T; 95°C, 30', 55°C, 40' for BRCA2-8765delAG). Each experiment was run in duplicate and 2 independent cDNAs were quantified. Standard calibration curves were also charted on serial dilutions of a cDNA, obtained from heterozygous samples corresponding to either the BRCA1-4446C-T or the BRCA2-8765delAG mutation under study, which was amplified by PCR and then purified with the QiAquick PCR Purification Kit (Qiagen Inc.). A solution of 2 mmol/L MgCl2, probes and primers, water, and 3 µL cDNA with 1:6 or 1:3 dilution was added in the 1 × SuperMix (Invitrogen Canada Inc.) to obtain a final volume of 15 µL.

**BRCA2-8765delAG**

Both alleles were quantified in competition. The final mix (15 µL) contained 0.2 µmol/L of BRCA2 Fw and Rev20Q primers and 0.2 µmol/L of BRCA2P20wt-LNA and P20mutated-LNA probes.

**BRCA1-4446C-T**

Both alleles were quantified in competition. The final mix (15 µL) contained 0.2 µmol/L of BRCA1 Fw and Rev1314-LNA primers and 0.1 µmol/L of BRCA1P1314wt-LNA and P1314mutated-LNA probes.

**TBP**

TBP was quantified in a separate reaction mix, with 0.2 µmol/L of each TBP primer and probe.

**Results**

**Identification of DEPS**

We compared the molecular profiles associated with either a BRCA1 (Fig. 1A) or BRCA2 (Fig. 1B) mutation between familial NOSEs (F-NOSEs) and nonfamilial NOSEs (NF-NOSEs), between BRCA2-mutated NOSEs (F-NOSE2) and BRCA1-mutated NOSEs (F-NOSE1; Fig. 1C), between BRCA1-mutated ovarian tumor cells (F-TOV1) and BRCA1-mutated NOSEs (F-NOSE1; Fig. 1D), and between BRCA1/2-mutated TOVs (F-TOV1-2) and BRCA1/2-mutated NOSEs (F-NOSE1-2; data not shown). Five probe sets (corresponding to 3 distinct genes) were found to be differentially expressed in F-NOSE1 versus NF-NOSEs (Fig. 1A), with 35 DEPs in F-NOSE2 versus NF-NOSEs (Fig. 1B). One hundred and thirty-three probe sets were differentially expressed in F-NOSE1 compared with NF-NOSEs (Fig. 1B). Corresponding significant genes are reported in Supplementary Table S1A to S1D. The molecular profiles obtained when comparing F-TOV1-2 with F-NOSE1-2 cells did not allow proper sample classification (data not included).

**Hierarchical clustering** based on DEPS was conducted for each comparison (Fig. 1, right charts). All comparisons revealed clear sample segregation corresponding to classes that were defined a priori for supervised analysis.

**Validation of the transcript levels of four candidate genes by q-RT-PCR**

To confirm the accuracy of our microarray results, we quantified the expression levels of a set of 4 candidate genes, selected for their biologic significance in carcinogenesis, by q-RT-PCR. Statistical significance was evaluated by Limma (linear model for microarray data analysis), and data on the corresponding probe sets appear in Supplementary Table S1.

As illustrated in Fig. 2, we found good overall concordance between the expression patterns obtained with the 2 quantification methods. Serum amyloid A2 (SAA2) transcripts corresponding to X51441_at and X51441_s_at (data not shown) probe sets were significantly upregulated in F-NOSE1 compared with NF-NOSEs (Fig. 2A; Supplementary Table S1 and S1A). Transcripts corresponding to J03474_at and X51441_at probe sets were significantly overexpressed in F-NOSE1 compared to F-NOSE2 (Fig. 2A; Supplementary Table S1 and S1C). Moreover, transcripts corresponding to 3 probe sets (J03474_at, X51441_at, and X51441_s_at; data not shown) were significantly downregulated in F-TOV1 compared with F-NOSE1 (Fig. 2A; Supplementary Table S1 and S1D). Both methods also quantified SAA1 transcript variants 1 and 2 on the positive strand (Supplementary Table S4A). Filamin A–interacting protein 1-like (FILIP1L) transcript corresponding to U53445_at was downregulated in F-NOSE2 compared with NF-NOSEs (Fig. 2B; Supplementary Table S1 and S1B). Annexin A8-like 2 (ANXA8L2) transcript corresponding to probe set X16662_at was overexpressed in F-TOV1 versus F-NOSE1 (Fig. 2C; Supplementary Table S1 and S1D). Finally, mannose phosphate isomerase (MPI) transcript measured by X76057_at was upregulated in F-NOSE2 compared with NF-NOSEs and F-NOSE1 (Fig. 2D; Supplementary Table S1 and S1D). Its expression was also significantly higher in F-TOV1 than in F-NOSE1 (Supplementary Table S1 and S1D) and this F-TOV1 overexpression was confirmed by q-RT-PCR (Fig. 2D).

**Quantification of BRCA1 and BRCA2 transcript levels by q-RT-PCR**

Quantification of BRCA1 and BRCA2 transcript levels by q-RT-PCR is shown in Fig. 3A and 3B. Interestingly, BRCA2 levels were low in F-NOSE2, with the exception of sample 12 (M2-12). The elevated BRCA2 transcript levels in sample 12 (Fig. 3B) were also associated with higher levels of BRCA1 (Fig. 3A), SAA2 (Fig. 2A), and FILIP1L (Fig. 2B) transcripts, compared with the other BRCA2-mutated samples. ANXA8L2 and MPI transcript levels were unaffected (Fig. 2C and D).

**Quantification of FILIP1L, SAA2, ANXA8L2, MPI, BRCA1, and BRCA2 by q-RT-PCR in a larger series**

Quantification of FILIP1L, SAA2, ANXA8L2, MPI, BRCA1, and BRCA2 by q-RT-PCR in a larger series is shown in Fig. 4.
Figure 2. A–D, expression level of 4 candidate genes (A, SAA2; B, FILIP1L; C, ANXA8L2; D, MPI) measured by q-RT-PCR and HuFL DNA microarray.

Top chart, q-RT-PCR results. Bottom chart, quantification using HuFL DNA microarray. The results showed generally good concordance between expression patterns obtained with the 2 methods, especially for NOSE samples. q-RT-PCR did not confirm overexpression of MPI in F-TOV1 observed with the HuFL DNA microarray. This discrepancy could be associated with an alternative splicing pattern in F-TOV1.
To strengthen the validity of our results, we increased our sample size by including additional independent donor primary cell lines. The characteristics of all donors and primary cell culture procedures are reported in Tables 1 (NOSEs) and 2 (TOVs). The quantification results are given in Fig. 4. Culture media were shown to have no effect on pattern specification (Fig. 4A–D). The results on BRCA2 (Fig. 4F) were neither significantly different between samples 16 (M2-16, cultivated in OSE standard medium) and 17 (M2-17, cultivated in MM), nor between samples 15 (M2-15) and 18 (M2-18), cultivated with MM according to the Kruk’s modified procedure. Important differences, relative to BRCA2 transcript levels were, however, observed between samples 12 (M2-12), 13 (M2-13), and 14 (M2-14), all cultivated in OSE standard medium (Fig. 4). Moreover, the variation of SAA2 transcript levels (Fig. 4A), as well as BRCA1 transcript levels seen in F-NOSE2 samples (Fig. 4E), was related to BRCA2 expression (Fig. 4F). In general, the expression profiles obtained in this larger series were concordant with initial HuFL array findings.

Quantification of wild-type and mutated allelic transcripts of BRCA2

To define the contribution of mutated transcripts to total expression level, we measured the expression of each allele in BRCA2-mutated samples (Fig. 5A). A subset of nonmutated samples served as controls to confirm the specificity of the mutated probe. Total BRCA2 levels were variable in morphologically normal nonmutated samples (Fig. 5A). For the 8765delAG mutation, we had not only F-NOSE2 samples but also a tumor sample, OV-552 (sample 22, TM2-22), and a tumor cell line TOV-81D (sample 23, TM2-23). Surprisingly, the wild-type allele was retained and expressed not only in morphologically normal samples, except for M2-12 donor which developed both breast and colonic cancer (Fig. 4F and Table 1). Moreover, the level of each allelic transcript was higher in tumor sample 22 (TM2-22), derived from aggressive tumor cells (OV-552) than in tumor sample 23 (TM2-23), derived from an indolent ovarian tumor cell line (TOV-81D; Fig. 5A). Ovarian cancer cells from the TOV-81D donor, despite originating from an invasive stage IIIC papillary serous ovarian adenocarcinoma, were well-differentiated (grade I). Their behavior in culture reflected those of an indolent tumor. The donor did not die of the ovarian tumor but of a second primary breast cancer diagnosed 12 years later. On the other hand, sample 22 (TM2-22) originated from cells isolated from the ascites produced by an aggressive grade III, stage IV serous papillary carcinoma. Whereas in TOV-81D (TM2-23), both allelic transcript levels were low, despite higher than in morphologically normal samples, they were overexpressed in OV-552 (TM2-22), in comparison not only to TOV-81D but also to F-NOSE2 and NF-NOSEs. It is interesting to notice that the mutated allele transcript level in sample 22 (TM2-22) is more than 120-fold higher than in sample 23 (TM2-23), indicating that the mutated transcript level could have influenced the clinical phenotype of the tumor (Fig. 5A).

Quantification of wild-type and mutated allelic transcripts of BRCA1

To define the contribution of each allelic transcript to total expression level, we measured the expression of each allele in BRCA1-mutated samples (Fig. 5B). We discovered that the wild-type allele was retained and expressed in every morphologically normal and tumor samples from carriers. A subset of nonmutated samples served as controls to confirm the specificity of the mutated probe. Heterozygosity of the sample used as a calibrator was verified by sequencing the corresponding q-RT-PCR product after a purification step (data not included).
Comparison with published results

An overview of relevant publications (BRCA1/2-mutated ovarian tumor data) appears in Supplementary Table S2. Most of these reports compared inherited tumors from BRCA-mutation carriers with sporadic tumors; others compared FH-IOSEs (immortalized cell lines) with NFH-IOSEs, few of them compared F-NOSEs with NF-NOSEs. Common genes between published and our results are listed in Supplementary Table S3. Thirteen of the genes listed in the inherited ovarian tumor literature (Supplementary Table S2) were present in our "F-TOV1 versus F-NOSE1" expression profile (Supplementary Table S1D). Nine of them, BCL2, ELF4, MPI, PAK2, PTSG2, TCF4, SAA2, SAA1, and WAS, were differentially expressed between "F-NOSE1 versus F-NOSE2" (Supplementary Table S1C). PTSG2, SAA2, and SAA1 were also part of the "F-NOSE1 versus NF-NOSEs" profile (Supplementary Table S1A), and MPI was identified in the "F-NOSE2 versus NF-NOSEs" expression profile (Supplementary Table S1B).

Figure 4. A–F, quantification by q-RT-PCR of 4 candidate genes transcripts on an extended series. By carrying out q-RT-PCR and analyzing the results with Pfaffl method on an extended series of 18 NOSEs and 5 F-TOVs, we were able to confirm the results obtained previously. The horizontal line on each figure represents the average ratio obtained with NF-NOSE samples for the corresponding transcript. Culture media had no effect on pattern specification. The main difference between samples M2-12 to 14, all cultivated with OSE standard medium, was related to the level of the BRCA2 transcript. BRCA2 transcript levels were generally lower in F-NOSE2 than in NF-NOSEs, except in sample M2-12, obtained from a carrier of a distinct BRCA2 mutation. Note that SAA2 and BRCA2 followed the same pattern of expression in F-NOSE2.
Allele-specific transcript quantification

**A**

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**B**

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<th>Mutated allele / TBP</th>
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Figure 5. Allele specific expression (ASE) measured by q-RT-PCR in BRCA2-mutated and nonmutated samples and analyzed with Pfaffi method. BRCA2-8765delAG amplicon (A; top chart). BRCA1-4446C>T amplicon (B; bottom chart). The wild-type allele was retained and expressed not only in morphologically normal but also in tumor samples. Partial transcript levels were significantly higher in TM2-22 extracted from OV-552 cells, originating from an aggressive and invasive ovarian adenocarcinoma, than in TM2-23, extracted from TOV-81D, a cell line derived from an indolent tumor. Moreover, a very high level of the mutated allele was measured in TM2-22 compared with that of TM2-23. Tumor aggressiveness in the former patient could result from a dosage effect of the mutated allele.

Discussion

The advent of DNA microarrays has enabled the identification of molecular signatures associated with BRCA1/2 mutations in morphologically normal tissues. However, because of reduced accessibility to such samples and limited amounts of available material, studies investigating molecular alterations associated with such mutations are rare and, for the most part, restricted to a small number of genes (12). Some of these studies include NOSEs obtained after prophylactic oophorectomy in women with a familial history (FH) of cancers but without any knowledge about the presence or absence of a familial mutation (FH-NOSEs). Others used IOSEs as a reference to identify ovarian tumor profiles according to their BRCA1/2 mutation status (27). Consequently, a portion of the genes identified thus far could reflect differences in the model systems deployed (tissue vs. culture), as we have shown previously (19). An extensive study with proteomic techniques was conducted to identify morphologically normal ovarian cell proteomes in IOSE cell lines, either F-IOSEs or NF-IOSEs (28). Although perfectly consistent, the IOSE model differs from ours in the immortalization of cell lines, which introduces another step in the carcinogenic process. IOSEs in culture also display a different morphology and phenotype compared with OSEs (12). Thus, the results presented here are unique and we are the first to identify molecular signatures associated with French Canadian BRCA1 and BRCA2 mutations in primary cultures of NOSEs. Thereafter, the Knudson team (15) published their findings showing an altered gene expression associated with various BRCA1/2 mutations not only on ovarian, but also on breast morphologically normal cells.

Interestingly, the expression profiles we identified in some samples were not only characterized by the presence of a BRCA2 mutation, but also by the level of BRCA2 transcript. Both wild-type and mutated allelic transcripts were expressed in all F-NOSE2 samples under study. Surprisingly, the wild-type allele was also retained and expressed in tumor samples obtained from BRCA2-8765delAG carriers. Until recently, it was thought that loss of heterozygosity (LOH) of BRCA1/2 was necessary for tumor transformation in breast and ovarian tumors. Previous reports showing the existence of LOH in these tumors were based mostly on either standard autoradiographic procedure with well-known limitations in data interpretation and/or semiquantitative fluorescence methods with arbitrary definition of the threshold for calling an allelic imbalance LOH. King and colleagues showed that loss of the BRCA1/2 wild-type allele was not required for BRCA1/2-linked breast carcinogenesis (29) but that LOH was present in their invasive epithelial ovarian cancer (EOC) cases. By developing a quantitative approach that uses allele-specific RT-PCR, Chen and colleagues found that allelic imbalance affecting BRCA1 and to a lesser extent BRCA2, may contribute to both familial and nonfamilial forms of breast cancer (30). The absence of LOH was previously reported in a germinai ovarian tumor developed in a BRCA2-6174delT mutation carrier, a histotype not considered to be part of the BRCA phenotype (31). Finally, the recent complete exome analysis of ovarian tumors by the Cancer Genome Atlas Research Network showed that 20% of the ovarian tumors were associated with a BRCA1 or BRCA2 mutation and that 11% lost BRCA1 expression through DNA hypermethylation (32).

Allele-specific expression (ASE) of BRCA2 was observed in pancreatic cancer cells from a BRCA2-2041insA donor (33). By measuring ASE in inherited ovarian adenocarcinoma from French Canadian BRCA1 and BRCA2 mutation...
carriers, we showed retention and expression of the wild-type allele in these tumors.

Taked together, these data suggest that heterozygosity for a BRCA1/2 mutation could be sufficient for the initiation of ovarian carcinogenesis. One of the alternative hypothesis could be that haploinsufficiency caused by a single mutant copy of a BRCA gene may reduce its dosage to a level that leaves morphologically normal cells from carriers susceptible to neoplastic transformation. In the presence of one functional BRCA copy, additional "hits" may still be required to initiate carcinogenesis. Some of the differentially expressed genes we identified, could be involved in this process, and warrants further investigations.

In the past, some TP53 mutants were shown to exhibit a transdominant phenotype (34). Similarly, the 8765delAG mutation could act as a dominant mutation. BRCA2 mRNA was previously found to be overexpressed in high-grade sporadic breast tumors (35) and sporadic EOCs (25). Moreover, we noted that the aggressiveness of our inherited ovarian tumors was associated not only with a high level of expression of the total BRCA2 transcript, as encountered previously in sporadic cancers, but also with a high level of expression of the mutated allele. A possible dosage effect, related to the expression level of BRCA2, especially of the mutated allele transcript, could determine the final penetrance of this gene and the aggressiveness of the associated tumor.

Among the differentially expressed genes we identified, several are good candidates that, in conjunction with a BRCA1/2 driver mutation, could be involved in ovarian carcinogenesis. SAA2, overexpressed in F-NOSE1 compared with NF-NOSEs, was significantly downregulated in F-TOV1 compared with F-NOSE1. The SAA2s are a family of genes and a pseudo-gene clustered on chromosome band 11p15.1 (36). SAA1 and SAA2, sharing 96% homology, code for acute-phase SAA (A-SAAs) apolipoproteins and are expressed in the epithelium of several normal tissues (37). A-SAAs have been identified as potential sera markers in lung (38), ovarian (39), and prostatic cancers (40). Interestingly, SAA1 gene expression was significantly altered in the macroscopically normal rectosigmoid mucosa from individuals with a family history of colon cancer (41). Our data suggest that these genes may also be involved in the early stages of inherited ovarian carcinogenesis. Theses genes have also been identified as candidate ovarian biomarkers in BRCA1 carriers by Bellacosa and colleagues (15).

FILIP1L was clearly downregulated in F-NOSE2 compared with NF-NOSEs. This gene, located on chromosome 3p12.1, has been implicated in angiogenesis inhibition (42). Initially named "Decreased in Ovarian Cancer" after its identification by a subtractive approach as a transcript that is downregulated in ovarian cancer cell lines compared with normal surface epithelial cells (43). FILIP1L was also found to be underexpressed in immortalized human prostate cells compared with senescent cells and to be lost in human prostate cancer cell lines and xenografts (44). Moreover, recent functional data from a chromosome transfer study suggested that the FILIP1L chromosomal region could be important in sporadic EOC suppression (45). Our data further corroborate these findings and indicate a potential role for the gene in early BRCA2-associated carcinogenesis.

Annexin A8-like 2 belongs to a multigene family of calcium- and phospholipid-binding proteins. These peptides share a common structural domain, the annexin core, which consists of 4 or more copies of annexin repeats, responsible for their calcium-regulated membrane-binding activity (46). Annexin A8 was shown to display phospholipid- and F-actin-binding properties, suggesting its involvement in the structural organization of actin-associated membrane domains (47). Its role in mammary gland involution has been shown in mice. In humans, Annexin A8 co-expresses with CK5 and CK17 in a basal cell–like subset of human breast carcinoma with poor survival. Moreover, 85.7% of BRCA1-related breast tumors co-expressed Annexin A8 and CK5 (46). Interestingly, our results indicate a putative association of Annexin A8-like 2 with ovarian tumor progression in BRCA1 carriers.

Finally, MPI purified in 1994 from the placenta codes for a relatively well-conserved 423 aa protein (48). Through its function in catalyzing the interconversion reaction between fructose-6-phosphate and mannose-6-phosphate, it plays a predominant part in the posttranscriptional maturation of several proteins. Mutated forms of the MPI gene are involved in inherited disorders, such as congenital disorder of glycosylation type lb (49). As early as the 1920s, Warburg showed a distinct biochemical phenotype in tumor cells that develops aberrant energy metabolism, by increasing activation of the glycolysis pathway to produce higher ATP levels. Key molecular events involved in the "Warburg effect" have to be further elucidated (50). MPI overexpression in cells could increase energy production and confer a selection advantage.

Our data support the existence of molecular signatures associated with BRCA1/2 mutations in NOSEs. Identifying these profiles is of great clinical importance, particularly when extensive searches for BRCA1/2 mutations in families come up empty. Proper profiling of patients with BRCA1/2 gene mutations in high-risk families will not only avoid bias in linkage and/or association studies aimed at identifying BRCA1, but also facilitate identification of patients at-risk. Furthermore, our data show that tumor cell phenotype could depend not only on the total level of BRCA2 expressed but also on the mutated allele transcript level, suggesting a role of ASE in the observed tumor cell phenotype. Some of our candidate genes follow a common pattern of expression with BRCA2 in BRCA2-mutated samples and could eventually be coordinately regulated in these samples. They may represent early changes associated with ovarian carcinogenesis in carriers and as a result be considered as putative target for chemoprevention. Finally, common regulatory genes and/or epigenetic factors, including the mutated allele itself, could alter the transcriptome and result in intrindividual differences in the clinical phenotypes encountered for a single driver mutation.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Cancer Prevention Research

Allelic Transcripts Dosage Effect in Morphologically Normal Ovarian Cells from Heterozygous Carriers of a BRCA1/2 French Canadian Founder Mutation

Diala Abd-Rabbo, Christine Abaji, Guillaume B Cardin, et al.

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